

n-3 and n-6 Fatty Acid Changes in the Erythrocyte Membranes of Patients with *Clostridium difficile* Infection

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The implications of circulating essential fatty acids (FA) on the inflammatory risk profile and clinical outcome are still unclear. In order to gain a deeper understanding of the role of polyunsaturated fatty acids (PUFA) in the pathogenesis of acute infection, we analyzed the FA content in red blood cell (RBC) membranes of patients with *Clostridium difficile* infection (CDI) and controls. We prospectively studied 60 patients including 30 patients with CDI and 30 controls to assess lipid concentrations in erythrocyte membranes using gas chromatography. We observed a higher level of saturated fatty acids (SFA) in RBC membranes from patients with CDI. In patients with CDI, we also noticed a higher level of 20:4 n-6 FA and only a small amounts of C20:2n-6, C20:3n-6 FAs, arachidonic acid (AA) precursors, which suggest an intense inflammatory reaction in the organism during infection. We also noticed low levels of n-3 FA in the RBC membranes of patients infected with CDI. There is a deficit of n-3 FA in patients with CDI. n-3 FA are probably used during CDI as precursors of pro-resolving mediators that may indicate a therapeutic role of n-3 PUFAs in CDI. The changes in fatty acids in erythrocyte membranes during CDI alter their functions which may have an impact on the clinical outcome.

Key words: *Clostridium difficile*, erythrocyte membrane, n-3 fatty acids, n-6 fatty acids, pathogenesis.

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Clostridium difficile infection (CDI) is a disease of the gastrointestinal tract caused by an anaerobic gram-negative rod- order: *Eubacteriales*, family: *Bacillaceae*, genus: *Clostridium*, species: *difficile*. The main symptoms of this infection include diarrhea of varying intensity accompanied by abdominal pain and fever (TEDESCO *et al.* 1974). Mortality in the course of CDI ranges from 6% to 38% depending on patient age or comorbid conditions (MITCHELL & GARDNER 2012). Unexpectedly, CDI has become one of the most prominent nosocomial infections of the past 20 years. Particularly disturbing is the increase in incidence, mortality, and recurrence of this infection. The pathogenesis of CDI is complex and still not fully understood, however a number of recent studies

have furthered scientific knowledge in this area (SURAWICZ 2013; GOUDARZI *et al.* 2014).

Lipids play an important biological role which goes far beyond their function as storage of energy. They are important intercellular messengers, they influence gene expression, and are also mediators that take part in the inflammatory reaction (NOWAK 2010; HOFMANOVA *et al.* 2014). Various derivatives of fatty acids (FA) are formed during the course of both physiological and pathological metabolic processes, often with different functions (HOFMANOVA *et al.* 2014). Polyunsaturated fatty acids (PUFA) are an important dietary element as they are synthesized from their precursors: 18:3n-3 and 18:2n-6 essential FAs by the human body (NOWAK 2010). The subsequent formation of further FA is carried out via down-

stream metabolic processes and depends on many factors such as the presence of lipid-influencing enzymes, cooperation of blood cells, endothelium, epithelium, or membrane receptors (HOFMANOVA *et al.* 2014). At this cellular and tissue level, host factors and environmental influence, such as diet, combines individually and leads to the diversity of each individual. The analysis of membrane lipid composition in red blood cell (RBC) provides a simple, suitable model for studying FA metabolism. Moreover some changes in the lipid layer of RBC membranes alter their fluidity which may contribute to clinical problems (MARTINELLI *et al.* 2008; SIENER *et al.* 2010; RAPHAEL & SORDILLO 2013; TAKKUNEN *et al.* 2013).

In an earlier study we noticed for the first time that acute infection of the gastrointestinal tract with *Clostridium difficile* (C. diff.) is associated with abnormalities in rheological properties of blood, as well as increased aggregation of RBCs, which correlated with severity of inflammation (CZEPIEL *et al.* 2014). Therefore we continued this study associated with potential erythrocyte membrane changes during CDI infection. We prospectively studied the FA content of RBC cell membranes in patients with CDI and controls.

Material and Methods

Abbreviations used in the text

AA, arachidonic acid; AI, Aggregation Index; BHT, butylated hydroxytoluene; BMI, body mass index; CDI, *Clostridium difficile* infection; C. diff., *Clostridium difficile*; COX-2, cyclooxygenase 2; DSS, dextran sulfate sodium; DHA, docosahexaenoic acid; EI, Elongation Index; EPA, eicosapentaenoic acid; FA, fatty acids; FAME, fatty acid methyl esters; IL-10, interleukin 10; HUS, hemolytic uremic syndrome; LA, linoleic acid; MUFA, monounsaturated fatty acids; PGE₂, prostaglandin E₂; PUFA, polyunsaturated fatty acids; RBC, red blood cells; SD, standard deviation; SFA, saturated fatty acids; UNSFA, unsaturated fatty acids.

Patients

The study was conducted in accordance with the Declaration of Helsinki (1975) and approved by the Local Ethics Committee (KBET/6/B/2014). We prospectively studied 60 patients. The study group included 30 patients with CDI (19 women and 11 men) hospitalized at the Department of Infectious Diseases, Jagiellonian University Hospital in Kraków, aged from 23 to 93 years (median:

64 years), with median body mass index (BMI) 24.3 kg/m² and 30 volunteers as control group (18 women and 12 men) from 32 to 85 years old (median: 61.3 years), with median BMI 24.0 kg/m². The diagnosis of C. diff. was based on history, epidemiological data, physical examination, and laboratory tests. CDI was diagnosed in patients experiencing diarrhea, defined as the passage of 3 or more unformed stools in 24 hours; the infection was then confirmed by the detection of the C. diff. antigen and toxins in feces using the C. diff. Quick Check Complete test kit (TechLab Inc., Blacksburg, USA). When the test came back positive for the antigen but negative for the toxin, the test for the C. diff. toxin was repeated with the ELISA C. diff. toxin A/B II test kit (TechLab Inc., Blacksburg, USA) or by genetic methods, Illumigene C. diff. (Meridian, Bioscience, Inc., Cincinnati, USA). All microbiological tests were performed in the Microbiology Department, University Hospital in Kraków. Patients were questioned about their diet. Each patient who actively modified his/her diet was excluded, for example: using energy drinks, abuse of alcohol, vegan/vegetarian diet, or diabetic diet. We also excluded patients on a reduction diet. All participants described their diet as typical. Exclusion criteria included the presence of any other acute or chronic inflammatory diseases in both groups and diarrhea from any reason in controls.

Sample collection

Venous blood samples were collected in K₂-EDTA-containing tubes. The first sample was taken no later than 48 hours after initiating therapy, when the therapeutic effect of the antibiotic was not yet fully realized and patients still manifested clinical symptoms.

Erythrocytes were separated from plasma by centrifugation (1500 × g, for 10 min) and washed with phosphate buffer saline. 10 µl of 0.05 % butylated hydroxytoluene (BHT) was added to each sample.

Reagents used for gas chromatography

BHT, 14 % BF₃ and fatty acid methyl esters (FAME) standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade chloroform, methanol and n-hexane were obtained from Merck (Darmstadt, Germany). Gases for chromatography of 5.5 purity level were purchased from AirLiquide (Poland).

Isolation of cell membranes from erythrocytes.

Hemoglobin-free RBC membranes were prepared by hypotonic hemolysis at 4°C in 10 mM Tris with pH=7.4. Membranes were then isolated by centrifugation (10.000 x g; 15 min) and washed several times to eliminate hemoglobin residues according to the method proposed by Graham (GRAHAM 2006).

Lipid analysis

Total lipid extraction from erythrocyte membranes was carried out with a solution of chloroform/methanol (2:1). The synthesis of FA methyl esters of total lipids in the erythrocyte membranes was carried out with 14 % BF₃ in methanol. An internal standard, heptadecanoic acid, was used throughout. The FA methyl esters were analyzed using gas chromatography (Agilent 6890N). Chromatograph parameters were as follows: FID 260°C, oven – start at 140°C and ramp up to 240°C. Column DB – 23 (60 m, 0.25 µm). Inlet temperature 250°C, split 40:1, injection 1 µl. FA methyl esters were identified according to standards (Sigma-Aldrich, Supelco, USA). The data were analyzed using ChemStation and Excel software. Results were expressed in relative percentage of total FA. We calculated total saturated FA (SFA), monounsaturated FA (MUFA), n-6 and n-3 PUFA, as well as *trans*-FA. Additionally, we calculated the n-3/n-6 ratio (GDULA-ARGASIŃSKA *et al.* 2015).

Statistics

All data are presented as means ± standard deviation (SD), or medians and interquartile ranges. Differences between study groups were determined using one-way or multi-factor analysis of variance including ANOVA with the Scheffé *post hoc* test. Potential confounding factors such as age, sex and BMI were included along with FA in the multi-factor model.

Analysis of similarities between content of the FA in RBC membranes of controls and CDI patients was also performed using clustering methods. Ward's method with a Euclidean distance matrix was used to group a set of objects in such a way that objects in the same group are more similar to each other than to those in other groups. Cluster analysis is a convenient multivariate method which aims to classify grouping objects of similar kind into respective categories on the basis of a set of measured variables. Calculations were performed using StatSoft, Inc. (2011), STATISTICA, version 10 statistical package software, and statistical significance was defined as $\alpha < 0.05$.

Results

In all patients with CDI, elevated temperature (37.5-39.8°C) and diarrhea were observed. On the day of admission, patients had an average of 6 bowel movements per day and they all complained of either abdominal pain or weakness. Singular episodes of vomiting (one to three) occurred in a group of 5 patients. We excluded patients with the severest CDI with poor prognosis, so none of our patients died. 11 patients were treated with metronidazole, 11 with vancomycin, and 8 patients were treated initially with metronidazole, but due to intolerance or lack of proper improvement, they were switched to vancomycin therapy. The mean time of hospitalization after CDI diagnosis was 10.1 days (SD±4.2 days).

Both groups had normal weight BMI (18.5-25 kg/m²), similar ages, and a typical Western diet. RBC FA content in patients with CDI and controls are shown in Table 1. After adjustment for age, sex and BMI we did not observe significant changes between adjusted and unadjusted multi-factor models.

The erythrocyte membrane SFA index (45.3%) was higher and MUFA index (27.9%) was lower in patients with CDI infection compared to the control group (SFA 41.5%, MUFA 30.4%). Total RBC n-6 acids were significantly higher in the CDI group compared to the control group (23.3% and 16.9%, respectively, $P=0.0001$).

Total n-3 FA were significantly decreased in the erythrocyte membranes of patients with CDI as compared to the control group (4.5% and 10.4%, respectively, $P=0.0001$). In the CDI group, we observed small amounts of docosahexaenoic acid (DHA). Eicosapentaenoic acid (EPA) content was lower (1.0%) when compared to the control group (3.6%, $P=0.0001$). The n-3/n-6 ratio was significantly decreased (three-fold) in patients with CDI (ratio 0.2) compared to the control group (ratio 0.6).

To determine the covariant behavior of the measured variables, we analyzed variables to identify which best discriminate the FA profile in erythrocyte membranes from controls and patients with CDI (Fig. 1). Variables were separated into three unique clusters. In the RBC of cluster 1 control group, 14:0, 16:1, 14:1, 15:0, 18:3 n-3, EPA and 20:4n-6 FAs were present. Cluster 2 grouped long-chain FAs (20:2n-6, 20:3n-6, 22:2n-6, 20:3n-3 and DHA). In cluster 3, the following FA variants grouped together: 16:0 and 18:1 (Fig 1, top). The distribution of variants was different in the RBC of CDI patients when compared with the control group. In cluster 1, 14:0, 14:1, 15:0 and n-3 FAs were dominant (18:3n-3, 20:3n-3 and EPA). The cluster 2 grouping included 16:1, 18:0 and 18:2n-6. The cluster 3 group included 16:0, 18:1 and 20:4n-6 FAs (Fig. 1, bottom).

Table 1

Erythrocyte membrane fatty acids profile as a [%] of peak area in the controls (n=30) and patients with CDI (n=30). Means \pm SD (min-max).

Fatty acids	Control	CDI	P
C14:0 myristic	3.0 \pm 2.0 (0.5-8.9)	3.7 \pm 2.1 (0.8-2.8)	P=0.01
C15:0 pentadecanoic	2.7 \pm 1.0 (0.6-3.5)	3.1 \pm 1.1 (1.0-3.2)	
C16:0 palmitic	22.5 \pm 9.7 (3.2-49.2)	28.1 \pm 8.9 (19.0-27.0)	
C18:0 stearic	13.3 \pm 6.8 (7.7-38.6)	10.4 \pm 4.6 (6.2-13.6)	
Total SFA	41.5 \pm 10.3(25.6-54.0)	45.3 \pm 10.6 (29.5-53.5)	
C14:1n-5 myristoleic	2.6 \pm 1.0 (0.5-5.7)	1.8 \pm 0.6 (0.2-4.3)	P=0.001
C16:1c palmitoleic	2.8 \pm 1.6 (0.6-4.2)	6.0 \pm 2.3 (3.3-6.8)	
C18:1n-9c oleic	25.0 \pm 11.0 (13.8-29.4)	20.1 \pm 8.5 (14.2-27.0)	
Total MUFA	30.4 \pm 11.5 (14.7-40.5)	27.9 \pm 8.2 (18.5-44.6)	
C18:2n-6 linoleic	7.6 \pm 3.4 (2.9-7.8)	6.1 \pm 2.3 (4.3-8.2)	P=0.0001
C20:2n-6 cis 11,14eicosadienoic	0.9 \pm 0.2 (0.2-1.4)	0.1 \pm 0.0 (0.0-0.3)	
C20:3n-6 dihom-?-linolenic	1.3 \pm 0.6 (0.2-1.9)	0.1 \pm 0.0 (0.0-0.8)	
C20:4n-6 arachidonic	6.1 \pm 1.9 (4.8-10.2)	16.9 \pm 5.4 (5.9-23.8)	
C22:2n-6 cis-13,16-docosadienoic	1.0 \pm 0.5 (0.6-1.8)	0.1 \pm 0.0 (0.0-0.5)	
Total PUFA n-6	16.9 \pm 6.0 (5.9-18.1)	23.3 \pm 6.4 (11.6-35.1)	P=0.0001
C18:3n-3 linolenic	3.9 \pm 0.9 (1.7-8.4)	0.7 \pm 0.5 (0.0-1.4)	P=0.0001
C20:3n-3 cis11,14,17 eicosatrienoic	1.3 \pm 0.4 (0.4-1.9)	2.7 \pm 0.7 (0.0-2.9)	P=0.0001
C20:5n-3 EPA	3.6 \pm 0.7 (1.4-5.7)	1.0 \pm 0.4 (0.1-1.8)	P=0.0001
C22:6n-3 DHA	1.6 \pm 0.5 (0.5-2.6)	0.1 \pm 0.0 (0.0-0.7)	P=0.0001
Total PUFA n-3	10.4 \pm 2.8 (8.3-15.4)	4.5 \pm 1.4 (2.0-6.2)	P=0.0001
n-3/n-6	0.6 \pm 0.1 (0.5-0.9)	0.2 \pm 0.1 (0.0-0.3)	P=0.0001

FA, fatty acids; MUFA, sum of mono-unsaturated FAs; n-3, sum of n-3 FAs; n-6, sum of n-6 FAs; n-3/n-6 FAs ratio; PUFA, polyunsaturated fatty acids; SFA, sum of saturated FAs; UNSFA, sum of unsaturated FAs.

Discussion

The impact of PUFA on the pathogenesis of several chronic diseases has been studied, including cardiovascular diseases, rheumatoid arthritis, neurodegenerative, and neoplastic diseases (CALVIELLO *et al.* 2013). The basis of our study was to assess whether and how acute inflammation affects the lipid composition of the cell membrane. Hypothetically, this could have two basic implications: FA could actively influence the course of acute inflammation, which has so far been demonstrated only in the course of chronic inflammatory processes, and the change in lipid composition of erythrocyte membrane could be translated into their proper functioning.

The acute inflammatory response is an organism's defense to a damaging stimulus, the delicate homeostasis between defensive pro-inflammatory and suppressive anti-inflammatory responses, is the basis of a successful reaction against an invading pathogen (HOFMANOVA *et al.* 2014). The immune system uses both local and systemic mechanisms in its response, both of which are extremely important, however the local response occurring at the site of injury allows for a quick defensive response. FA as a component of cell membranes are almost always and immediately present at the inflammatory site (CHAPKIN *et al.* 2007; HOFMANOVA *et al.* 2014). The effect of n-6 FA mainly results from the action of arachidonic acid (AA) and their derivatives, and it is mainly

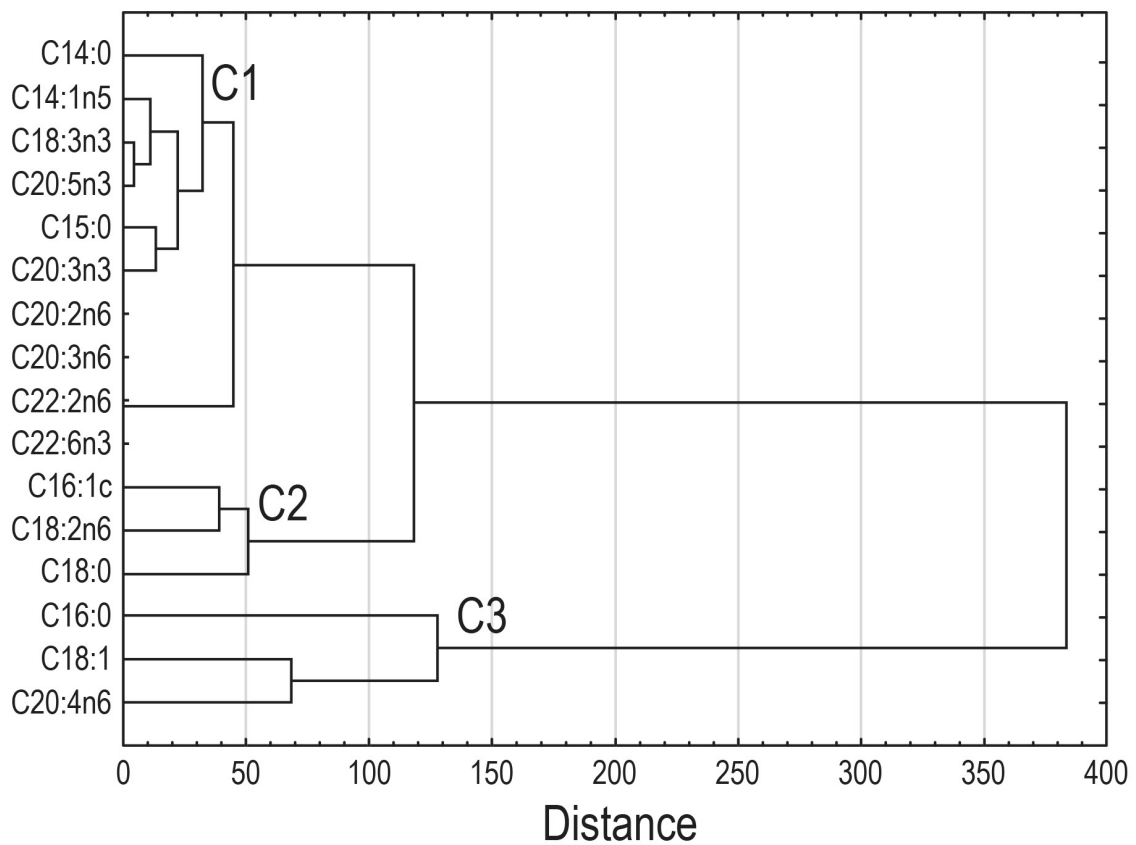
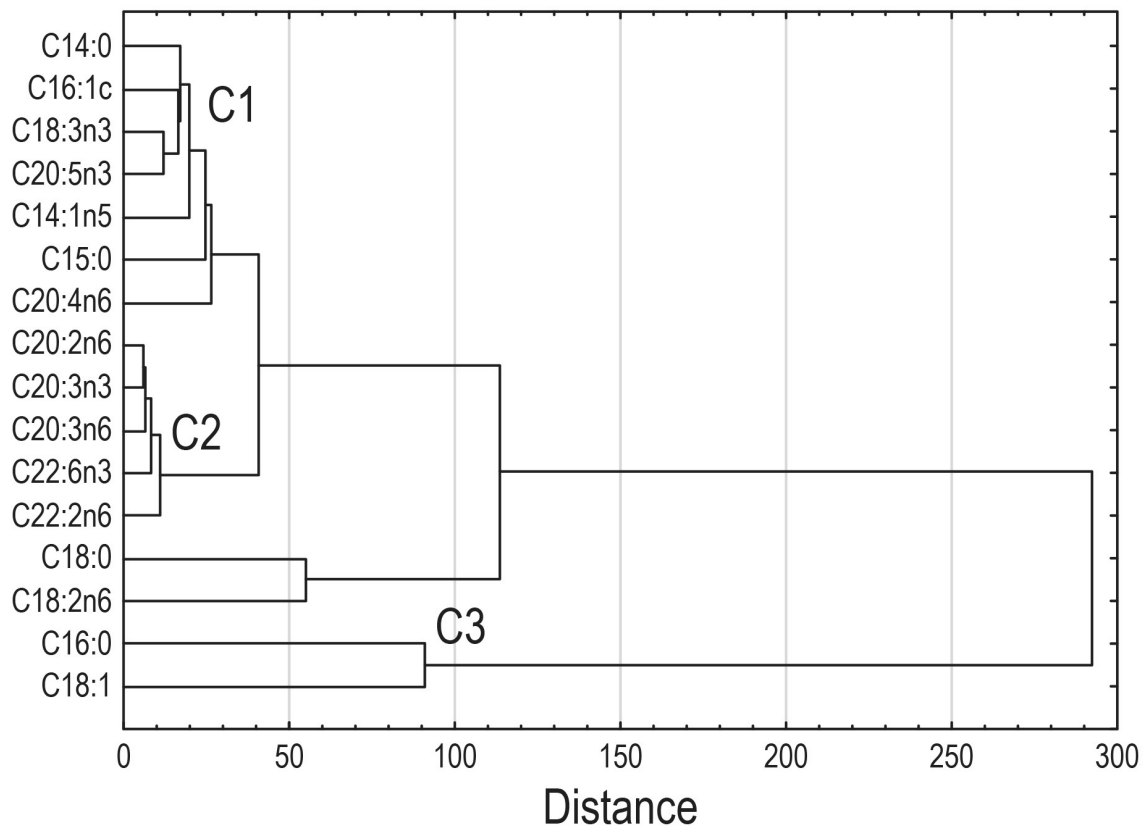


Fig. 1. Cluster analysis of the FA profile in RBC membranes from controls (top) and from CDI patients (bottom). Data were segregated into 3 unique clusters of variables by hierarchical cluster analysis.

pro-inflammatory. Conversely, n-3 FA display strong anti-inflammatory and pro-resolving effects, particularly the newly discovered resolvins and protectins which work as “stop-signals” arresting the inflammatory process (KHAN *et al.* 2014; GDULA-ARGASIŃSKA *et al.* 2015).

Some disorders with a more pronounced and clearly-defined inflammatory character such as chronic obstructive pulmonary disease, asthma and Crohn’s disease have become more prevalent in most Western societies in the past decades, in parallel with a greatly increased intake of n-6 PUFA. Clear associations between the risk of their onset and a shifted balance of n-6 PUFA and n-3 PUFA intake are lacking, but many investigators have recognized the potential of long-chain n-3 PUFA in dampening excessive inflammation in most inflammatory diseases and conditions (SIJBEN & CALDER 2007).

To the best of our knowledge, our study is the first that assesses FAs in acute infectious colitis among humans, and current scientific knowledge of the influence of PUFA on acute colitis comes only from animal studies. It was demonstrated that substitution of linoleic acid (LA) with long chain n-3 PUFA protected the colonic mucosa in dextran sulfate sodium (DSS) induced colitis (TYAGI *et al.* 2014). It was also shown that endogenously produced n-3 PUFA in transgenic fat-1 mice, decreases cyclooxygenase 2 (COX-2) and prostaglandin E₂ (PGE₂) expression, thus fulfilling a protective role in DSS-colitis (GRAVAGHI *et al.* 2011). As mentioned above some studies showed the protective effect of n-3 PUFA in experimental colitis, but also a few studies have failed to show this. For example it was demonstrated that a diet with 6% fish oil and 1% corn oil (n-6/n-3 ratio 0.17) aggravated colitis (WOODWORTH *et al.* 2010). In mice, a diet rich in n-6 PUFA strengthened the immunologic response during *E. coli* infection (WAN & GRIMBLE 1987), whereas a diet rich in n-3 FA but with a decreased amount n-6 decreased the intensity of colitis in interleukin 10 (IL-10) knockout mice (MANE *et al.* 2009). It appears as though this game of appropriate ratio of n-3/n-6 has a significant impact on the ideal response to a stimulus between the strong initial inflammatory response and its subsequent arrest. However a diet rich in n-3 PUFA, via its anti-inflammatory effect, may have a negative effect on the antimicrobial response. n-6 PUFA augments colitis, but prevents infection-induced systemic inflammation. In contrast, n-3 PUFA supplementation reverses the effects of the n-6 PUFA diet but impairs infection-induced responses resulting in sepsis (GHOSH *et al.* 2013).

In our study, we observed significant changes in the FA profile of erythrocyte cell membranes in

patients with CDI. The most significant changes were found in n-6 and n-3 FA, which suggests an intense inflammatory reaction within an organism during infection. We observed less than half the level of n-3 FA in patients with CDI compared to patients in the control group and a small amount of DHA. This may suggest a deficit of n-3 FA in the bodies of patients with CDI. This allows us to hypothesize that anti-inflammatory n-3 FA may serve as pro-resolving mediators during CDI infections. The n-3/n-6 ratios in RBC membranes were significantly lower (three-fold) in patients with CDI than in the control. This lower ratio in CDI patients is a reflection of ongoing inflammation in these patients and indicates the role of PUFA in the inflammation.

The subsequent question is how, in the acute inflammatory process, such a quick and dramatic change such as the change in membrane lipid composition seen in our study could occur. Mature red cells cannot synthesize membrane lipids *de novo*. Therefore, lipid exchange and acylation of FA is the only known possible mechanism for phospholipid repair and renewal. The exchange rate of outer bilayer phospholipids with the phospholipids of lipoproteins in plasma is slow (a turnover time of 5 days) while phospholipids in the inner leaflet of the bilayer are essentially nonexchangeable. However free cholesterol in red cell membranes exchanges readily with the unesterified free cholesterol in plasma lipoproteins (a half-life of 7 h) (CHAPKIN *et al.* 2007). Taking into account that the blood of patients with CDI in our test group was taken at the beginning of the infection, the change in the RBC cell membranes observed in this study must be different from the classically described renewal of membrane lipids. This warrants further study.

The statistically different FA compositions of RBC membranes may play a role in rheological patterns of RBC during CDI. In an earlier study, we observed significant changes in the hemorheologic characteristics of RBC. Alterations in RBC membranes are one of the important factors responsible for these characteristics. We found a statistically significant increase in Aggregation Index (AI) and alterations in RBC Elongation Index (EI) in the group of patients with CDI (CZEPIEL *et al.* 2014). The FA pattern of membrane phospholipids is suggested to affect membrane fluidity and epithelial barrier function as a result of membrane FA unsaturation. RBC membranes are composed of 35.1% lipids, when disregarding water content this is 43.6% lipids (YAWATA 2003). The state of saturation of FA, and the length of acyl chains affect the degree of membrane fluidity. When the degree of desaturation increases, the packing of

hydrophobic tails in the core of the bilayer is disrupted. Differences in lipid composition may have important implications for the membrane properties of the RBC. The significant changes in FA observed in our study might be one of the factors important in RBC deformability changes during acute infection. The shape of RBC, which also depends on the state of the membrane lipids, is another important factor influencing deformability. Strongly charged amphipathic compounds i.e. phospholipids, can cause echinocytosis due to their trapping in the outer leaflet of the lipid bilayer by a fixed charge (YAWATA 2003).

Oxidative stress in the hyperinflammatory state during CDI may also cause RBC membrane changes, including cytoskeleton rearrangement as well as oxidation and loss of lipid asymmetry. Moreover, RBCs exposed to both endogenous and exogenous sources of reactive oxygen and nitrogen species undergo not only mechanical alteration such as decreased deformability but also accumulation of oxidative damage products such as lipid oxidation products (BUTTARI *et al.* 2015). For example Shiga toxin-producing *Escherichia coli* infections cause hemolytic uremic syndrome, characterized by thrombotic microangiopathy that leads to hemolytic anemia (GOMEZ *et al.* 2013). Hemolytic uremic syndrome (HUS) often follows infectious diarrhea. A variety of enteric pathogens including *Salmonella species*, *Escherichia coli* O157:H7, and *Campylobacter jejuni* are causative agents of HUS (KESHTKAR-JAHROMI & MOHEBTASH 2012). The association between 658240462 *Clostridium difficile* and HUS is not as obvious. There are a few cases reported from 1977 which indicate that HUS is a possible, but very rare, complication of CDI (TOCHEN & CAMPBELL 1977; MBONU *et al.* 2003; KESHTKAR-JAHROMI & MOHEBTASH 2012; ALVARADO *et al.* 2014). The mechanism of this process is not understood but it is known that *C. diff.* toxins, due to Rho inactivation, disrupt the cytoskeleton (GOUDARZI *et al.* 2014). We hypothesize that *C. diff.* is capable of hemolytic impacts of varying severity, with consequent damage to the erythrocyte membrane and subsequently leads to the observed disturbances in the lipid composition of RBCs. Only in small numbers of cases is this mechanism severe enough to cause the development of HUS, which would explain the small number of CDI-associated HUS cases reported.

Taken together, oxidative stress may contribute to the senescence and oxidation of RBCs. This suggests that in patients with CDI, the lifetime of the erythrocytes might be reduced either by oxidative stress or by increased cell hemolysis.

In conclusion, erythrocyte membrane FA composition in CDI patients is reported for the first

time in this study. We discovered a significant decrease in the levels of n-3 FA as well as a significant increase in n-6 FA in patients with CDI. Because the membrane FA content effects membrane fluidity, these findings are worthy of further investigation. The deficit of n-3 FA in RBC cell membranes in patients with CDI may suggest a potential therapeutic role of n-3 PUFAs; that they hypothetically could be used as nutraceuticals to modify the composition and organization of cell membranes to selectively initiate a change in their fluidity and function. The data obtained may be useful in further studies of the biochemistry of lipids in patients with acute infection.

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